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Identification of Common and Distinct Residues Involved in the Interaction of α_{i2} and α_s with Adenylyl Cyclase*

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The G protein α subunits, α_s and α_{i2}, have stimulatory and inhibitory effects, respectively, on a common effector protein, adenylyl cyclase. These effects require a GTP-dependent conformational change that involves three α subunit regions (Switches I-III). α_s residues in three adjacent loops, including Switch II, specify activation of adenylyl cyclase. The adenylyl cyclase-specifying region of α_{i2} is located within a 78-residue segment that includes two of these loops but none of the conformational switch regions. We have used an alanine-scanning mutagenesis approach within Switches I-III and the 78-residue segment of α_{i2} to identify residues required for inhibition of adenylyl cyclase. We found a cluster of conserved residues in Switch II in which substitutions cause major losses in the abilities of both α_{i2} and α_s to modulate adenylyl cyclase activity but do not affect α subunit expression or the GTP-induced conformational change. We also found two regions within the 78-residue segment of α_{i2} in which substitutions reduce the ability of α_{i2} to inhibit adenylyl cyclase, one of which corresponds to an effector-activating region of α_s. Thus, both α_{i2} and α_s interact with adenylyl cyclase using: 1) conserved Switch II residues that communicate the conformational state of the α subunit and 2) divergent residues that specify particular effectors and the nature of their modulation.

Upon activation by cell surface receptors, heterotrimeric G proteins transmit signals to effector proteins that regulate a wide variety of cellular processes (1–4). Receptors activate G proteins by catalyzing the replacement of GDP bound to the α subunit with GTP, resulting in dissociation of α·GTP from the βγ subunits. The GTPase activity of the α subunit regulates the timing of deactivation and reassociation of the G protein subunits. The fidelity of cellular signaling requires that α subunits modulate effector proteins only when bound to GTP and that only the appropriate α subunit-effector pairs interact. GTP-dependent effector interaction most likely involves one or more of the three α subunit regions that change conformation during the GTPase cycle (Switches I-III), identified by comparison of the x-ray crystal structures of the GTPγS-bound[1] (active) and GDP-bound (inactive) forms of α_t (5, 6) and α_{i1} (7, 8). Differences in the amino acid sequences of the structurally conserved α subunits (40% identity at the amino acid level, with 60–90% identity within subfamilies) determine the specificity and nature of their interactions with effector proteins (9). However, the relationship between the molecular determinants of effector specificity and of GTP-dependent effector regulation is poorly understood.

Regulation of adenylyl cyclase by the G protein α subunits, α_s and α_i, raises issues specific for this α subunit-effector interaction. α_s and α_i, which are relatively poorly conserved among the family of α subunits (~40% identical amino acids), both bind to adenylyl cyclase but have opposite effects on activity. Inhibition of adenylyl cyclase by α_i requires prior activation by α_s, forskolin, or calmodulin (10, 11). Since adenylyl cyclase can be inhibited by α_i in the absence of α_s, inhibition does not appear to be due to competition between α_i and α_s for binding to adenylyl cyclase. Indeed, there is evidence that suggests that adenylyl cyclase has distinct binding sites for α_s and α_i (11). Key questions that arise are: why does α_s activate and α_i inhibit, and why do only α_s and α_i, but not other α subunits, modulate adenylyl cyclase activity?

The α_s residues that specify activation of adenylyl cyclase are located in three adjacent loops, one of which includes Switch II (12). The location of a conformational switch region within the effector-specifying surface of α_s provides a simple mechanism for the GTP-dependence of the α_s-adenylyl cyclase interaction. However, studies with chimeric α subunits containing portions of α_{i2} and α_q, which does not interact with adenylyl cyclase (13), showed that an $\alpha_q/\alpha_{i2}/\alpha_q$ chimera containing only 78 residues of α_{i2} (residues 245–322) inhibits adenylyl cyclase as well as α_{i2} does (14). This 78-residue effector-specifying segment includes residues homologous to two of the three clusters of α_s residues that specify activation of adenylyl cyclase (12, 15) but does not include any of the conformational switch regions. This was a surprise since the GTP-bound form of α_i is much more effective at inhibiting adenylyl cyclase than the GDP-bound form is (11). However, the importance of the conformational switch regions might have been missed using a chimeric α subunit approach due to the high degree of sequence similarity in these regions between α_q and α_{i2}.

To determine whether any of the conformational switch regions are involved in inhibition of adenylyl cyclase by α_{i2}, we substituted alanines for solvent-exposed residues in these regions. We tested the effect of these mutations on both the inhibition of adenylyl cyclase and the ability of the mutant proteins to achieve the activated conformation as measured by the acquisition of trypsin resistance upon binding of GTP. We identified a part of Switch II that is conserved among α subunits in which alanine substitutions blocked the inhibition of adenylyl cyclase by α_{i2}. We also found that substitutions of alanines for the corresponding α_s residues specifically prevent activation of adenylyl cyclase. Thus it appears that both α_{i2} and

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[1] The abbreviations used are: GTPγS, guanosine 5′-*O*-(thiotriphosphate); hGH, human growth hormone; PDE, cGMP phosphodiesterase; PLC, phosphoinositide phospholipase C.

α_s interact with adenylyl cyclase using two types of residues: 1) conserved residues within Switch II that signal that the α subunit is in the GTP-bound active conformation and 2) divergent residues that specify activation or inhibition of this effector enzyme.

To identify the α_{i2} residues involved in specifying inhibition of adenylyl cyclase, we substituted alanines for solvent-exposed residues within the 78-residue segment. We found two regions of sequence in which mutations impaired the ability of α_{i2} to inhibit adenylyl cyclase, the amino terminus of α3 and the α4/β6 loop. The α4/β6 loop is also important for the effector interactions of α_s (12) and α_t (16, 17). These substitutions did not cause as much of a decrease in adenylyl cyclase inhibition as the Switch II mutations did, suggesting that Switch II residues are the primary contributors to the interaction between α_{i2} and adenylyl cyclase.

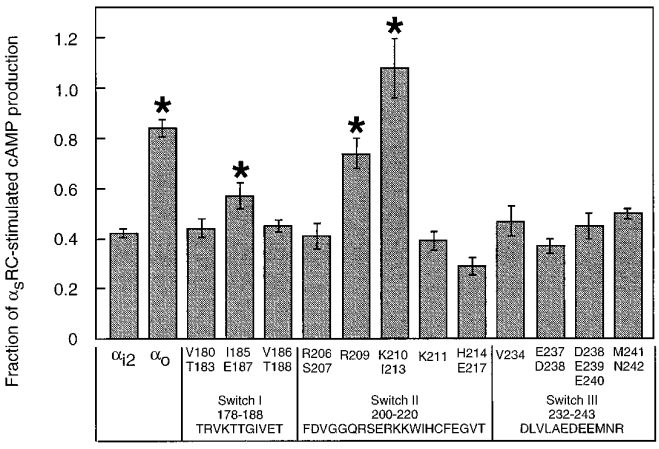


FIG. 1. **Alanine substitutions of solvent-exposed residues in Switches I-III.** The residues that were substituted by alanines in each construct and the residue ranges and sequences of Switches I-III in α_{i2} are indicated. All constructs include the GTPase-inhibiting arginine to cysteine mutation (R179C in α_{i2} and α_o) and the EE epitope. cAMP accumulation in 10^6 HEK-293 cells transfected with 0.1 μg of vector containing α_sRC and 0.3 μg of vector containing the indicated α subunit constructs is shown. The amount of cAMP accumulation in cells transfected with α_sRC alone is set at 1.0, and the values from cells co-transfected with the indicated constructs are expressed relative to this value. *Asterisks* indicate cAMP values of constructs with significantly decreased abilities to inhibit cAMP accumulation ($p < 0.05$) compared with α_{i2}RCEE. cAMP levels in [^{3}H]adenine-labeled cells were determined as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least three independent experiments.

EXPERIMENTAL PROCEDURES

Generation of Plasmids—α_{i2} mutants were constructed from the mouse α_{i2} cDNA (18), and α_s mutants were constructed from the rat α_s cDNA (19). Two modifications were made to each of the α subunits to facilitate detection of their activities and expression levels. The arginine at position 179 in α_{i2} and 201 in α_s was mutated to cysteine to inhibit GTPase activity and produce constitutive activation (20, 21). An epitope, referred to as the EE epitope (22) was generated by mutating α_{i2} residues SDYIPTQ (166–172) to EEYMPTE and α_s residues DYVPSD (189–194) to EYMPTE (single letter amino acid code, mutated residues are underlined). The resultant constructs were designated α_{i2}RCEE and α_sRCEE respectively. α_oRCEE was generated from the rat α_o cDNA (19) by mutating arginine 179 to cysteine and residues DYQPTE (167–172) to EYMPTE.

The α_{i2}RCEE cDNA (gift of Ann Pace and Henry Bourne, University of California, San Francisco) was subcloned into pcDNA I/Amp (Invitrogen) as an *Eco*RI fragment. The α_sEE cDNA (gift of Paul Wilson and Henry Bourne, University of California, San Francisco) was subcloned into pcDNA I/Amp as a *Hin*dIII fragment. To produce the α_sRC cDNA, the α_sRCHA cDNA (12), which contains the HA epitope from influenza virus (23), was digested with *Xba*I and *Eco*RI to yield a fragment containing the R201C mutation but not the HA epitope. *Xba*I-*Eco*RI restriction of α_sEEpcDNA I/Amp removed a fragment containing the EE epitope, which was replaced by the *Xba*I-*Eco*RI fragment from the α_sRCHA cDNA to produce α_sRCpcDNA I/Amp. To generate α_sRCEEpcDNA I/Amp, α_sRCpcDNA I/Amp was digested with Alwn I to yield a fragment containing the R201C mutation, which was ligated into α_sEEpcDNA I/Amp in place of the analogous fragment to produce an α_s cDNA containing both the R201C mutation and the EE epitope.

All mutations were generated by oligonucleotide-directed *in vitro* mutagenesis (24) using the Bio-Rad Muta-Gene kit except for those in the α_{i2}RCEE derivatives, Constructs 2 and 3, which were produced by polymerase chain reactions that generated DNA fragments with overlapping ends that were subsequently combined in a fusion polymerase chain reaction (25). All mutagenesis procedures were verified by restriction enzyme analysis and DNA sequencing.

cAMP Accumulation Assay— Recombinant α subunits were transiently expressed in the human embryonic kidney fibroblast line, HEK-293 (American Type Culture Collection CRL-1573), using DEAE-dextran (26) under the control of the cytomegalovirus promoter in the expression vector, pcDNA pcDNA I/Amp. To measure inhibition of adenylyl cyclase, 10^6 cells/60-mm dish were co-transfected with 0.1 μg of vector containing α_sRC and 0.3 μg of vector containing α_{i2}RCEE, α_oRCEE, or mutant derivatives of α_{i2}RCEE. To measure activation of adenylyl cyclase, 10^6 cells/60-mm dish were transfected with 1.5 μg of vector containing α_sRCEE or mutant derivatives of this construct or with vector alone. Intracellular cAMP levels in cells labeled with [^{3}H]adenine were determined as described (14).

Membrane Preparations and Trypsin Assay—HEK-293 cells were transiently transfected with recombinant α subunit constructs using DEAE dextran (26). Membranes were prepared 48 h after transfection as described (14). For the trypsin resistance assay (12), membrane proteins (70 μg) were diluted to a concentration of 6 mg/ml in a buffer containing 20 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 2 mM β-mercaptoethanol, and 0.64% (w/v) of the detergent lubrol PX. Solubilized proteins were collected after centrifugation for 10 min at 4 °C in a microcentrifuge and incubated for 30 min at 30 °C in the presence or absence of 125 μM GTPγS. Tosylphenylalanyl chloromethyl ketone-treated trypsin (Sigma T-8642) was added to a final concentration of 5 μg/ml, and the mixture was incubated for 5 min at 30 °C. The digestion was terminated by adding soybean trypsin inhibitor to a final concentration of 1 mg/ml. The samples were then resolved by SDS-polyacrylamide electrophoresis (10%), transferred to nitrocellulose, and probed with the anti-EE monoclonal antibody (22), which was purified from hybridoma supernatants using E-Z-SEP reagents (Middlesex Sciences, Inc). The antigen-antibody complexes were detected using an anti-mouse horseradish peroxidase-linked antibody according to the ECL Western blotting protocol (Amersham Life Science, Inc.).

RESULTS

Characterization of Mutant α_{i2} Constructs Using cAMP Assay—To characterize mutant α_{i2} subunits after transient expression in HEK-293 cells, two features were included, as in a previous study (14), to enable measurement of their functions without interference from the activities of the α_i proteins endogenous to these cells. First, a conserved arginine (R179C) was replaced by cysteine. This mutation constitutively activates α_{i2} by inhibiting its GTPase activity (20) and made it possible to measure inhibition of adenylyl cyclase without requiring receptor-mediated activation of the mutant α_{i2} subunits. Second, the α_{i2} constructs include an epitope from an internal region of polyoma virus medium T antigen, referred to as the EE epitope (22), which does not interfere with the α_{i2}-adenylyl cyclase interaction (27).

We measured the ability of recombinant α subunits to inhibit adenylyl cyclase in HEK-293 cells by co-expressing them with the constitutively activated α_s mutant, α_sRC, in which arginine 201 is mutated to cysteine (21). As in a previous study (14), transfection with 0.1 μg of vector containing α_sRC resulted in an approximately 18-fold increase in cAMP production compared with cells transfected with vector alone. Co-transfection with 0.3 μg of vector containing α_{i2}RCEE resulted in ~60% inhibition of the cAMP response to α_sRC, while co-transfection with the same amount of vector containing α_oRCEE inhibited the response to α_sRC by only ~15% (Fig. 1). We used α_oRCEE as a negative control because α_o has been shown to have little or no ability to inhibit adenylyl cyclase (10, 11).

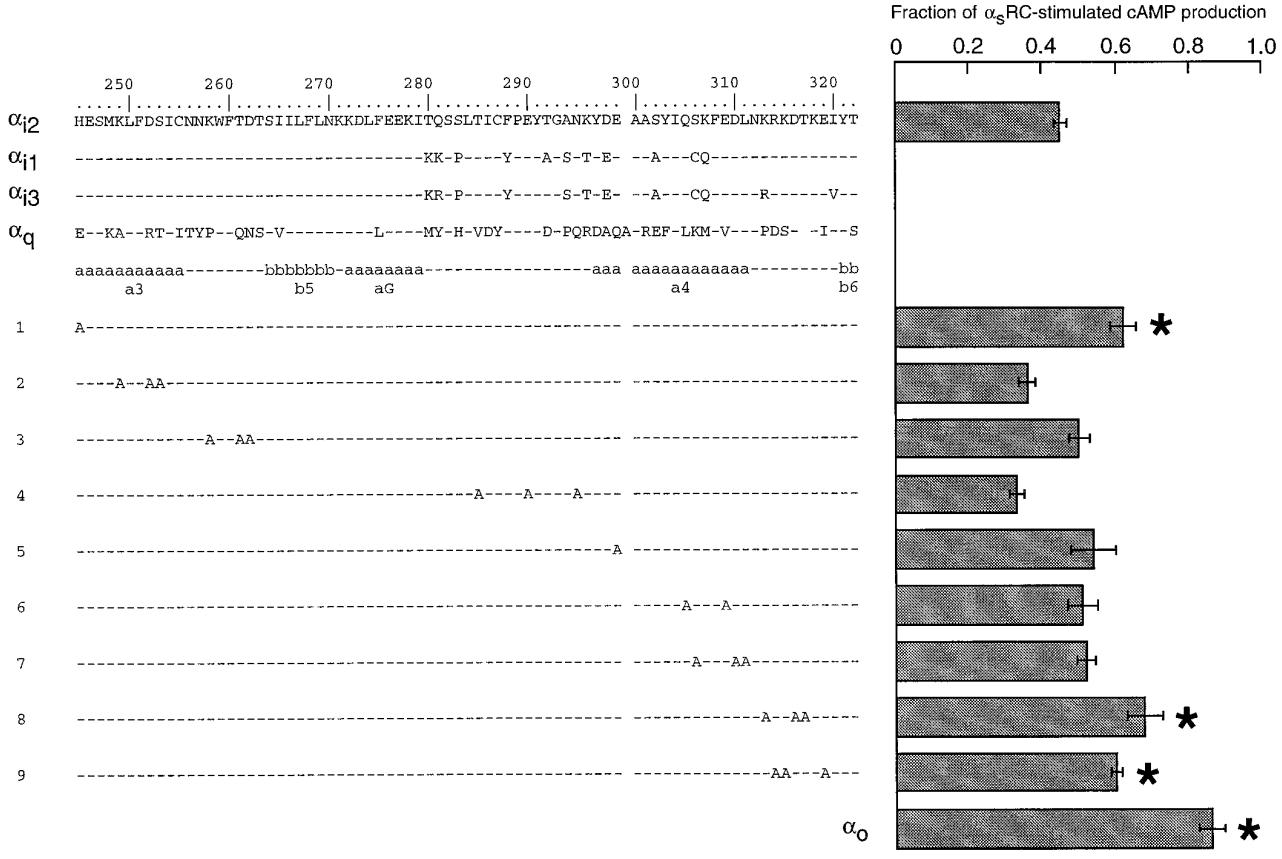
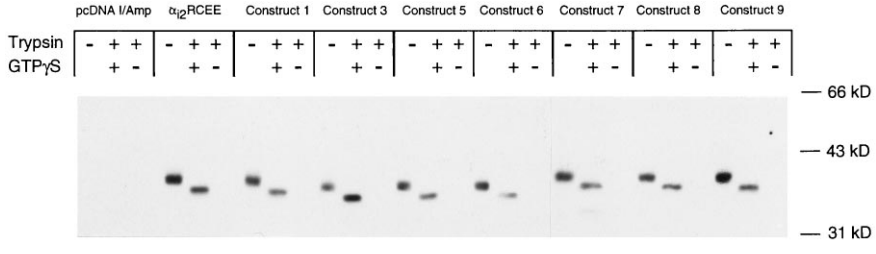


FIG. 4. **Alanine substitutions of solvent-exposed residues within the 78-residue α_{i2} segment.** The *top sequence* is that of α_{i2} residues 245–322. Below that are the sequences of α_{i1}, α_{i3}, and α_q, with residues identical to α_{i2} residues represented by *dashes*. The *numbered sequences* represent individual mutant constructs with alanine substitutions at the indicated positions. All constructs include the GTPase-inhibiting arginine to cysteine mutation (R179C in α_{i2} and α_o) and the EE epitope. Shown next to each construct is the cAMP accumulation in 10^6 HEK-293 cells transfected with 0.1 μg of vector containing α_sRC and 0.3 μg of vector containing the indicated α subunit construct. The amount of cAMP accumulation in cells transfected with α_sRC alone is set at 1.0, and the values from cells co-transfected with the indicated constructs are expressed relative to this value. *Asterisks* indicate cAMP values of constructs with significantly decreased abilities to inhibit cAMP accumulation ($p < 0.05$) compared with α_{i2}RCEE. cAMP levels in [^{3}H]adenine-labeled cells were determined as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least three independent experiments.

FIG. 5. **Expression and trypsin sensitivity of α_{i2} constructs containing mutations within the 78-residue α_{i2} segment.** 12.5×10^6 HEK-293 cells were transfected with 2 μg/10^6 cells of vector alone or vector containing the indicated α_{i2} constructs, and membranes were prepared, treated with trypsin, and immunoblotted as described under "Experimental Procedures." The *first lane* in each set is the control (no trypsin). The *second* and *third lanes* show the result of trypsin digestion in the presence or absence, respectively, of GTPγS.



criteria, residues Arg-209, Lys-210, and Ile-213 are specifically required for interaction with adenylyl cyclase. In contrast, although the Switch I mutant construct, (I185A,E187A)α_{i2}-RCEE, exhibited resistance to trypsin in the presence of GTPγS, it was expressed very poorly (Fig. 2). The role of residues Ile-185 and Glu-187 in effector interaction is, therefore, uncertain.

In the course of these studies, we mutated the α_{i2} residue, Arg-209, that corresponds to the GTPγS-protected trypsin site determined by amino-terminal sequencing of tryptic peptides from α_t and α_o (31). Elimination of this cleavage site would be expected to result in an α subunit that was resistant to trypsin cleavage in both the presence and absence of GTPγS. However, (R209A)α_{i2}RCEE was resistant to trypsin cleavage in the presence but not the absence of GTPγS (Fig. 2). Similar results were obtained upon mutation of each of the other potential trypsin sites in Switch II, Arg-206,[2] Lys-210 (Fig. 2), and Lys-211,[2] as well as mutation of all four residues simultaneously.[2] These results suggest that, although Switch II may contain cleavage sites that change conformation upon GTP binding, there are also other sites outside of this region that are preferentially cleaved by trypsin in the absence compared with the presence of GTPγS. Nevertheless, the ability of the trypsin assay to detect GTP-dependent conformational changes in Switch II is demonstrated by the fact that the Switch II α_s mutant, G226Aα_s, which is unable to undergo the activating conformational change required for dissociation from $\beta\gamma$,

[2] C. H. Berlot, unpublished observations.

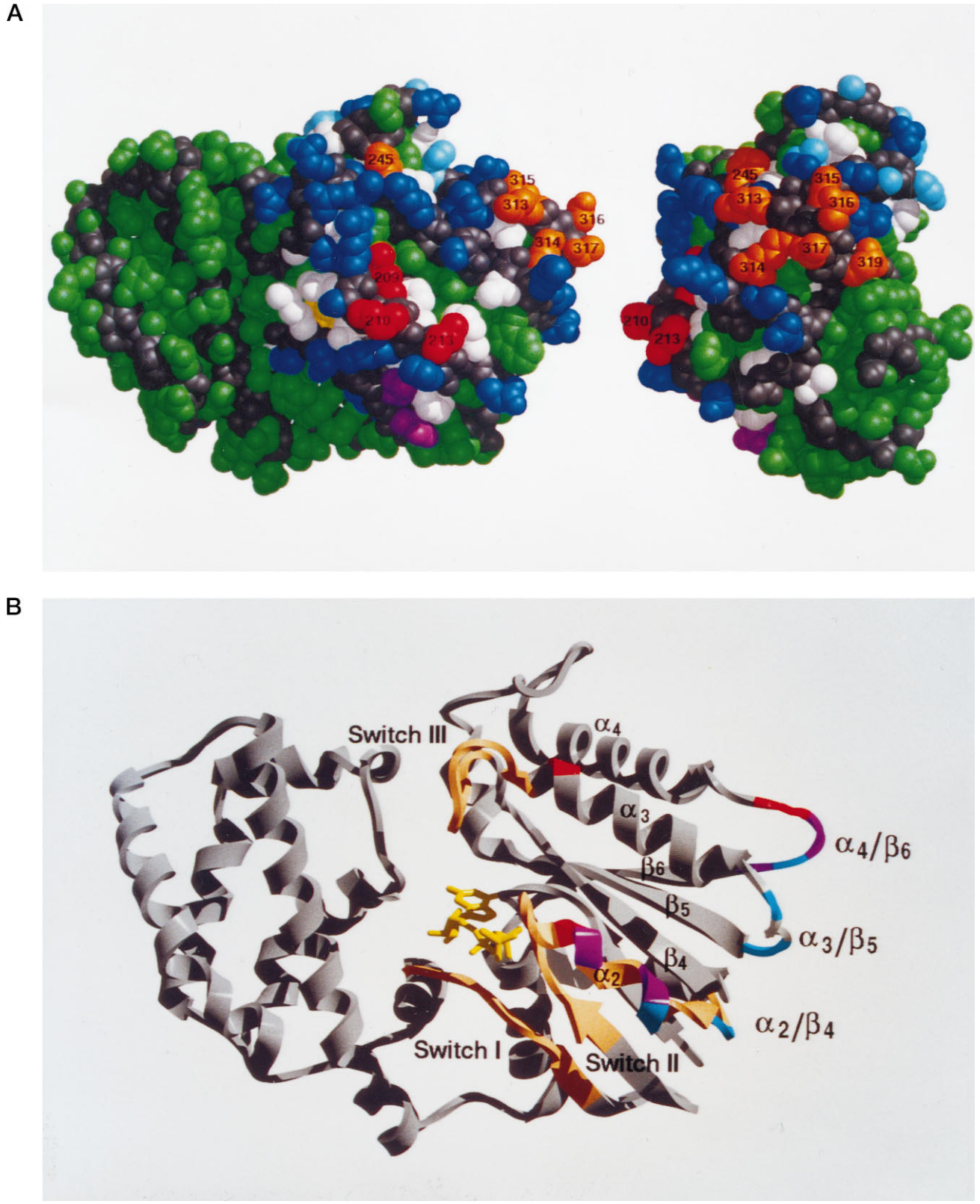


FIG. 6. **Mapping of effector-interacting residues of α_{i2} and α_s onto the x-ray crystal structure of the GTPγS-bound form of α_{i1}.** *A*, space-filling model showing α_{i2} residues required for inhibition of adenylyl cyclase. Residues that were mutated are shown in *red*, *magenta*, *orange*, and *dark blue*, as follows. Residues in Switch II specifically required for inhibition of adenylyl cyclase are *red*. Residues in Switch I in which mutations reduce both inhibition of adenylyl cyclase and expression level are *magenta*. Residues within the 78-residue segment in which mutations cause a partial loss of adenylyl cyclase inhibition are *orange*. Residues in which mutations do not affect inhibition of adenylyl cyclase are *dark blue*. Residues that were not mutated in this study are shown in *green*, *light blue*, and *white*, as follows. Residues outside of the 78-residue segment and Switches I-III are *green*. Also *green* are the residues within the 78-residue α_{i2} segment that are conserved between α_{i2} and α_q. The α_{i2} residues within this segment that differ from α_q residues but are not identical among the three α_i isoforms are *light blue*. Residues in Switches I-III that were not mutated and residues within the 78-residue segment that differ from α_q residues and are conserved among the three α_i isoforms, but were not mutated, are *white*. Main chain backbone atoms are *gray*. The GTP is *yellow*. The *numbers* on the model refer to α_{i2} residues. The model on the *right* is rotated approximately 90° about the vertical axis relative to the model on the *left*. *B*, ribbon diagram showing comparison of

does not acquire trypsin resistance in the presence of GTPγS (32, 33).

A Conserved Region of Switch II Is Specifically Required for the Effector Interactions of Both α_s and α_{i2}—To determine whether the highly conserved middle region of Switch II (see Fig. 7) is required for the activation of adenylyl cyclase by α_s, we tested the effects of substituting alanines for the α_s residues (Arg-232 and Ile-235) that correspond to Lys-210 and Ile-213 in α_{i2}. We introduced these substitutions into α_sRCEE, which contains the EE epitope, previously shown to have no effect on the interaction between α_s and adenylyl cyclase (34). The substitutions almost entirely prevented α_sRCEE from activating adenylyl cyclase without affecting the GTP-dependent conformational change measured by the trypsin assay (Fig. 3). Thus, the same region of Switch II is required for the interaction of both α_s and α_{i2} with adenylyl cyclase.

Alanine Substitutions within the 78-Residue Segment—Since α_{i2}, but not α_q, inhibits adenylyl cyclase (10, 13) and an $\alpha_q/\alpha_{i2}/\alpha_q$ chimera containing only 78 α_{i2} residues (245–322) inhibits adenylyl cyclase as well as α_{i2} does (14), the α_{i2} residues that specify inhibition of adenylyl cyclase must be located within this 78-residue segment. To identify these effector-specifying residues, we tested the effects of mutating nine clusters of solvent-exposed residues (22 residues total) to alanine residues (Fig. 4). Within the 78-residue segment of α_{i2}, 65 residues are identical among the three α_i isoforms, which have equal abilities to inhibit adenylyl cyclase (11). Of these 65 residues, 28 are different in α_q and therefore might account for the ability of α_i, but not α_q, to inhibit adenylyl cyclase. 20 of the substitutions were in residues that are identical among the three α_i subunits, and 18 were in residues that differ between α_{i2} and α_q. The thoroughness of our mutational analysis is illustrated in Fig. 6*A*.

As shown in Fig. 4, substitutions of three sets of residues: His-245 (Construct 1), Lys-313, Asp-316, and Thr-317 (Construct 8), and Arg-314, Lys-315, and Glu-319 (Construct 9), significantly reduced inhibition of adenylyl cyclase. However, in contrast to the Switch II mutations, which entirely blocked the ability of α_{i2}RCEE to inhibit adenylyl cyclase, the mutations in Constructs 1, 8, and 9 had only partial effects. The other six clusters of mutations (15 residues) did not significantly impair the ability of α_{i2}RCEE to inhibit adenylyl cyclase.

All of the constructs that inhibited adenylyl cyclase to a similar or decreased extent compared with α_{i2}RCEE were expressed in HEK-293 cell membranes and were able to undergo the GTP-dependent conformational change that results in increased resistance to trypsin digestion (Fig. 5). However, since scanning densitometry of immunoblots showed that Constructs 1, 8, and 9 were expressed at lower levels than α_{i2}RCEE was, their decreased abilities to inhibit adenylyl cyclase may be due to effects of the mutations on protein folding and/or stability. Nevertheless, since we have substituted alanines for the majority of solvent-exposed residues within the effector-specifying 78-residue segment (see Fig. 6*A*) and the other substitutions did not significantly reduce adenylyl cyclase inhibition, the residues in Constructs 1, 8, and 9 are, by default, the most likely candidates for specifying inhibition of adenylyl cyclase.

Comparison of the Effector-Interacting Surfaces of α_{i2} and α_s—We used the x-ray crystal structure of the GTPγS-bound form of α_{i1} (7) to map the results of our mutagenesis studies. 88% of the residues in α_{i2} can be aligned with identical residues in α_{i1}, while 67% of the α_{i1} residues can be aligned with identical residues in α_t. Since the structures of the active (GTPγS-bound) forms of α_{i1} (7) and α_t (5) are virtually identical, the structure of α_{i1} is an excellent model for that of α_{i2}. Our mutagenesis analysis of Switches I-III in α_{i2} and the 78-residue effector-specifying α_{i2} segment, residues 245–322, focused on solvent-exposed residues. In addition, most of the alanine substitutions in the 78-residue segment were of residues that are: 1) different from the homologous α_q residues and 2) conserved among the α_i isoforms. The thoroughness of this study is demonstrated by the fact that the residues in Switches I-III that were not mutated and the residues in the 78-residue segment that meet criteria 1 and 2 but were not mutated represent a very small fraction of the available surface area (shown in *white* in Fig. 6*A*).

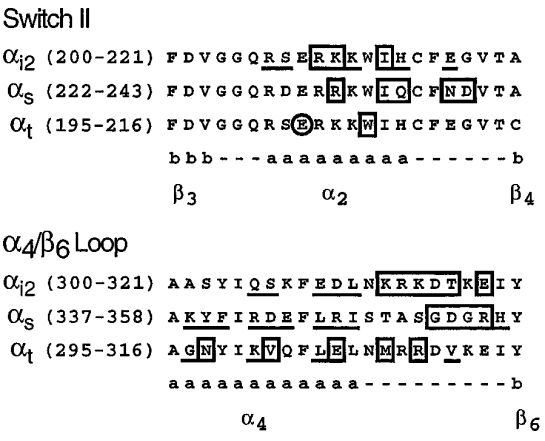


FIG. 7. **Comparison of effector-interacting residues of α_{i2}, α_s, and α_t in Switch II and in the $\alpha4/\beta6$ loop.** Residue numbers of α_{i2}, α_s, and α_t in the Switch II and $\alpha4/\beta6$ regions are indicated in parentheses. Mutations of *boxed* residues impaired effector interaction. Mutations of *underlined* residues did not impair effector interaction. Mutation of the *circled* glutamate residue in Switch II of α_t caused constitutive activation of PDE. Data for α_{i2} are from Figs. 1 and 4. Data for α_s are from Fig. 3 and Berlot and Bourne (12). Data for α_t are from Spickofsky *et al.* (17), Faurobert *et al.* (36), and Mittal *et al.* (37).

The alanine substitutions that caused the largest decrease in the ability of α_{i2}RCEE to inhibit adenylyl cyclase were in the middle of the $\alpha2$ helix in Switch II (*red* in Fig. 6*A*). The effector-interacting surfaces of α_s and α_{i2} overlap exactly in this region (*magenta* in Fig. 6*B*) where the sequences of the two α subunits are highly conserved (Fig. 7). However, the $\alpha2/\beta4$ loop at the carboxyl-terminal end of Switch II is important for the interaction of α_s (12) but not α_{i2} (Fig. 1) with adenylyl cyclase (*blue* in Fig. 6*B*).

The alanine substitutions within the 78-residue effector-specifying segment that caused a moderate reduction in the ability of α_{i2}RCEE to inhibit adenylyl cyclase (*orange* in Fig. 6*A*) were in the amino terminus of $\alpha3$ (Construct 1) and in the $\alpha4/\beta6$ loop (Constructs 8 and 9) (Fig. 6*B*). The amino terminus of $\alpha3$ (*red* in Fig. 6*B*) is important for the effector interactions of α_{i2} (Fig. 4), but not α_s (12), while mutations in the $\alpha3/\beta5$ loop (*blue* in Fig. 6*B*) disrupt interaction between α_s and adenylyl cyclase (12) but do not have a significant effect on the α_{i2}-adenylyl cyclase interaction (Fig. 4). Residues in the $\alpha4/\beta6$ loop found to be important for specifying the effector interactions of both α_{i2} and α_s are *magenta* in Fig. 6*B*.

effector-interacting surfaces of α_{i2} and α_s. Residues important for the effector interactions of α_{i2} are *red*, of α_s are *blue*, and of both α subunits are *magenta*. Switches I-III are *orange*. The model is in the same orientation as the model on the left in *panel A*. Coordinates of the GTPγS-bound form of α_{i1} are from Coleman *et al.* (7). The figures were drawn using MidasPlus, developed by the Computer Graphics Laboratory at University of California, San Francisco.

DISCUSSION

The studies reported here investigated two key aspects of α subunit-effector interactions, GTP-dependence and specificity. We found that in the case of α_{i2}, these two components of effector interaction are mediated by distinct regions of surface residues. GTP-dependent effector interaction is mediated by Switch II residues that are conserved among α subunits (Fig. 1) while specificity (inhibition of adenylyl cyclase) is mediated by nonconserved residues (the amino terminus of α3 and the α4/β6 loop) outside of the conformational switch regions (Fig. 4). In contrast, in the case of α_s, Switch II plays a role in regulating both the GTP dependence of effector interaction as well as effector specificity. The conserved Switch II region is required for GTP-dependent activation of adenylyl cyclase (Fig. 3) while nonconserved Switch II residues, as well as residues outside of the conformational switch regions (the α3/β5 and α4/β6 loops), are involved in regulating effector specificity (12). In the case of α_t, the conformational switch regions and regions that don't switch conformation (α3 and the α3/β5 loop) interact with distinct regions of the effector molecule, PDE (35).

Taken together, our results and those of others indicate that two α subunit regions, Switch II and the α4/β6 loop, may be important for effector interactions in general (Fig. 7). The conserved middle region of Switch II has been shown to be important for the interaction between α_t and PDE. Mutation of a conserved tryptophan in α_t reduces binding to PDE (36) while mutation of a conserved glutamate causes constitutive activation of PDE by the GDP-bound form of α_t (37). The α4/β6 loop is involved in specifying the effector interactions of at least three α subunits (Fig. 7). We previously found that replacement of α_s residues in this region by their α_{i2} homologs prevents α_s from activating adenylyl cyclase without preventing the mutant protein from attaining the GTP-dependent active conformation (12). Rarick *et al.* (16) found that a 22-amino acid peptide (α_t residues 293–314) activates PDE. Within this region, Spickofsky *et al.* (17) identified five residues in which substitutions of homologs from other α subunits block PDE activation by peptides. Three of these residues are in the α4 helix and two are in the α4/β6 loop. Mutations in the α4/β6 loop of α_s and α_{i2}, but not in α4 cause decreases in effector modulation. In the case of α_q, α4 and the α4/β6 loop have been implicated in PLC activation in studies using peptides (38). However, chimera studies showed this region could be replaced with α_s sequence without affecting PLC activation (39).

Since α_s and α_{i2} have opposite effects on adenylyl cyclase activity, the conserved region of Switch II required for the effector interactions of both α subunits is most likely involved in regulating GTP-dependent effector binding. Of the three residues found to be important for inhibition of adenylyl cyclase by α_{i2}, Arg-209 and Ile-213 are identical in the sequences of α_s and α_{i2} (see Fig. 7). The third residue is conserved but not identical between the two α subunits (Lys-210 in α_{i2}, Arg-232 in α_s). However, α_{i2}/α_s chimera studies showed that substitution of lysine for arginine at position 232 in α_s has no effect on activation of adenylyl cyclase (12). Furthermore, the α_q residue corresponding to Lys-210 is an arginine residue and α_q/α_{i2} chimera studies showed that substitution of arginine at this position does not affect inhibition of adenylyl cyclase (14). Therefore, these Switch II residues do not determine the nature of adenylyl cyclase modulation by α_s and α_{i2}.

Although all α subunits are conserved in this Switch II region, other α subunits do not modulate adenylyl cyclase, with the exception of a weak inhibition of type I adenylyl cyclase by α_o (11). A possible explanation for this selectivity is that other α subunits contain residues that preclude a productive adenylyl cyclase interaction. If so, then replacing α_{i2} residues in the amino terminus of α3 and in the α4/β6 loop with the homologous residues from α_q or other α subunits might cause a larger reduction in ability to inhibit adenylyl cyclase than was observed for alanine substitutions.

Our studies show that the effector-specifying regions of α_s and α_{i2} overlap but are not identical (see Fig. 6*B*). Studies using α subunit chimeras localized the region of α_{i2} that specifies inhibition of adenylyl cyclase to a 78-residue segment (amino acids 245–322) that extends from α3 to β6 (14). Residues corresponding to two of the three α_s regions that specify activation of adenylyl cyclase (12, 15), the α3/β5 and α4/β6 loops, are included in this segment. The only region of overlap that we have found among the effector-specifying regions of α_s and α_{i2} is in the α4/β6 loop. Effector-specifying regions unique for α_s are located in the α3/β5 loop and in the carboxyl-terminal part of Switch II (12). Similarly, mutation of a single residue in the amino terminus of α3 reduces the ability of α_{i2} to inhibit adenylyl cyclase but is not required for the activation of adenylyl cyclase by α_s (12).

Since both α_s and α_{i2} interact with adenylyl cyclase, the effector-specifying residues of each α subunit presumably determine whether activation or inhibition will result from α subunit binding. However, the effector-specifying residues of α_s appear to contribute more to the interaction with adenylyl cyclase than do those of α_{i2}. Substitutions in the effector-specifying segment of α_{i2} do not cause as large of a decrease in the ability to inhibit adenylyl cyclase as do substitutions in the conserved middle part of Switch II. However, mutations in two of the effector-specifying regions of α_s, the nonconserved carboxyl-terminal part of Switch II and the α3/β5 loop, decrease effector activation to the same extent as do mutations in the conserved Switch II region.[2] Consistent with our results, Taussig *et al.* (11) found that replacing α_{i1} residues with α_s homologs in the α3/β5 loop results in an α subunit that weakly activates certain adenylyl cyclase isoforms. Thus, the effector-specifying regions of α_s appear to be dominant over those of α_i.

Mutagenesis studies of hGH and its receptor, for which a structure of the hormone-receptor complex is available (40), have characterized the functional importance of residues in the binding interface. Individual replacements of residues in hGH (41) and its receptor (42) demonstrated that only a small subset of the residues at the center of the contact region contribute substantially to binding affinity. However, hGH residues in the periphery of the interface, which do not contribute much to the affinity of binding (41), are important for the specificity of binding (43).

In a similar manner, our studies of the interaction between α_{i2} and adenylyl cyclase implicate Switch II residues as being the major contributors to this binding interaction. Substitutions in the effector-specifying segment of α_{i2} have a more modest effect on the ability of α_{i2}RCEE to inhibit adenylyl cyclase. In the absence of any structures of α subunit-effector complexes, we predict that interactions between these proteins will include the conserved Switch II region as well as nonconserved specificity regions but that, as seen in the case of hGH and its receptor (41, 42), the contact surfaces may be larger than the "functional epitopes" defined by our mutagenesis studies.

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